Regular Article

Increased Milk Protein Concentration in a Rehydration Drink Enhances Fluid Retention Caused by Water Reabsorption in Rats

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A fluid-retention effect is required for beverages that are designed to prevent dehydration. That is, fluid absorbed from the intestines should not be excreted quickly; long-term retention is desirable. Here, we focused on the effect of milk protein on fluid retention, and propose a new effective oral rehydration method that can be used daily for preventing dehydration. We first evaluated the effects of different concentrations of milk protein on fluid retention by measuring the urinary volumes of rats fed fluid containing milk protein at concentrations of 1, 5, and 10%. We next compared the fluid-retention effect of milk protein-enriched drink (MPD) with those of distilled water (DW) and a sports drink (SD) by the same method. Third, to investigate the mechanism of fluid retention, we measured plasma insulin changes in rats after ingesting these three drinks. We found that the addition of milk protein at 5 or 10% reduced urinary volume in a dose-dependent manner. Ingestion of the MPD containing 4.6% milk protein resulted in lower urinary volumes than DW and SD. MPD also showed a higher water reabsorption rate in the kidneys and higher concentrations of plasma insulin than DW and SD. These results suggest that increasing milk protein concentration in a beverage enhances fluid retention, which may allow the possibility to develop rehydration beverages that are more effective than SDs. In addition, insulin-modifying renal water reabsorption may contribute to the fluid-retention effect of MPD.

Key words milk protein; milk protein-enriched drink (MPD); fluid retention; water reabsorption; insulin

Humans regulate their body temperature by sweating and *via* the skin blood flow for the release of heat accumulated in the body by exercise and working in a warm environment. Sweating thus helps control body temperature efficiently, but it may also cause dehydration. Dehydration inhibits sweating and skin blood flow, which impairs the function of thermoregulation.[1] It is thus important that the net fluid balance in the body is maintained positively by adequate rehydration.

For the treatment of dehydration by rehydration drinks, rapid fluid absorption from the intestine is necessary. Today, sports drinks and oral rehydration solution (ORS) are used for this purpose. An ORS contains sodium and carbohydrates at the appropriate ratio, which enables the rapid absorption of the ORS fluid in the small intestine.[2] An ORS was reported to be effective for the treatment of the dehydration caused by diarrhea.[3]

In addition to rapid fluid absorption, fluid retention is required for rehydration drinks for the purpose of preventing dehydration. In other words, when a rehydration drink is used to prevent dehydration, the fluid absorbed from the intestine should not be excreted quickly as urine; it should stay in the body as long as possible. A primary goal of rehydration drinks is to prevent dehydration by providing a high fluid-retention effect before an individual works or exercises in a warm environment.

Previous studies have reported that the addition of sodium[4] or carbohydrates[5] to a drink enhances its fluid-retention effect, and the studies suggested that 50 mM sodium or 12% carbohydrate is necessary to provide a fluid-retention effect. These concentrations of sodium and carbohydrate are too high to be taken daily in a drink, because the excessive intake of sodium can cause high blood pressure, and that of carbohydrates can cause hypertriglyceridemia.[6,7]

Milk was reported to have a fluid-retention effect and to be effective for the treatment of post-exercise dehydration.[8,9] Milk protein is shown to have a higher fluid-retention effect than carbohydrate at the same weight.[10] A recent study in rats showed that milk protein accounts for 50% of the total fluid retention provided by milk.[11] We thus hypothesized that the addition of milk protein to a carbohydrate-electrolyte beverage could enable more effective rehydration for preventing dehydration.

In the present study, we focused on the effect of milk protein on fluid retention, and we propose a new effective oral rehydration method that can be used daily for preventing dehydration. Toward this end, we first evaluated the dose effect of milk protein on fluid retention. Second, we evaluated the fluid-retention effect of a milk protein-enriched drink (MPD); that is, a drink containing milk protein at an effective concentration, by comparing its effects with those of distilled water (DW) and a carbohydrate-electrolyte sports drink (SD). Third, we measured the plasma insulin changes after the ingestion of

Table 1. Compositions of the Experimental Drinks

Composition	Unit	SD	MPD
Protein	%	0.0	4.6
Fat	%	0.0	1.3
Carbohydrate	%	6.2	5.0
Sodium	mM	21.3	19.1
Potassium	mM	5.1	33.2
Calcium	mM	0.5	37.7

SD, sports drink; MPD, milk protein-enriched drink.

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these three drinks as a way to investigate the mechanism of fluid retention.

MATERIALS AND METHODS

Experimental Materials In Experiment 1, we used a cow's milk protein concentrate (MPC480; Fonterra Co-operative Group Ltd., Auckland, New Zealand) to make milk protein solutions that contained protein at 1, 5, or 10%. The milk protein concentrate is 77.2% protein, 1.2% fat, 9.2% carbohydrate, and 0.1% sodium.

To assess the contribution of the ingredients other than protein to the fluid-retention effect, we made a solution in which protein was eliminated by ultrafiltration of the 5% milk protein solution mentioned above. For the ultrafiltration, a filter with the nominal molecular weight limit of 3000 Da (Sartorius Stedim Biotech, Goettingen, Germany) was used. The protein concentration of the ultrafiltrate was measured by the bicinchoninic acid method (Thermo Fisher Scientific, Wilmington, DE, U.S.A.), and the results confirmed that the protein removal rate was 95%.

In Experiments 2 and 3, we used a conventional protein-free sports drink and a milk protein-enriched drink (Meiji Co., Ltd., Tokyo). The compositions of these experimental drinks are shown in Table 1.

Animals Male 7-week-old Sprague-Dawley rats were purchased from Japan SLC (Shizuoka, Japan). The rats were maintained at 21±2°C with relative humidity of 55±15% with a 12-h light-dark cycle (light cycle: 07:00–19:00). The rats were fed a standard chow, Charles River Formula-1 (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Each experiment was carried out after a >1-week acclimation period.

This study was approved by the Animal Committee of the Food Science Research Laboratory, Meiji Co., Ltd., and the animals' care was in accord with the guidelines laid down by this committee.

EXPERIMENTAL DESIGN

Experiment 1: Relationship between the Milk Protein Concentration and the Fluid-Retention Effect In Experiment 1, we evaluated the fluid-retention effect of the solutions by measuring the volume of the rats' urine excretion after an oral administration of each solution, as described.[11] Briefly, the rats were starved for 20 h and were deprived of water for 4 h followed by an oral administration of 6 mL of one of the test solutions *via* a stomach sonde. The test solutions were distilled water (DW), 1, 5, and 10% milk protein solution (MP), and ultrafiltrate of the 5% MP. Immediately after the solution administration, the rats were placed in individual wire netting cages. Their urine was collected and the urine volume was measured every 30 min for 4 h.

Experiment 2: The Fluid-Retention Effect of the Milk Protein-Enriched Solutions By the same method as that used in Experiment 1, we measured the volume of urine excreted after an oral administration of DW, SD, or MPD.

Blood was collected from the rats' saphenous vein at 0, 2, and 4 h after the administration of each test drink to measure the hematocrit and hemoglobin by automatic analysis equipment (XT-1800i; Sysmex, Kobe, Japan). We calculated the percentage changes in the plasma volumes with the hematocrit and the hemoglobin at 0 and 4 h after oral administration.[12] After the last blood collection, the rats were laparotomized under isoflurane anesthesia. The stomachs and small intestines were extracted and the digesta in them were gathered. The digesta were freeze-dried, and the weights of the water remaining in the lumens after 4 h were measured.

Plasma was collected from the remaining blood sample. Creatinine in the plasma and the urine were measured by Fuji Drychem (FUJIFILM, Tokyo, Japan) and LabAssay™ Creatinine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

The rate of water reabsorption was calculated with the following equation.[13]

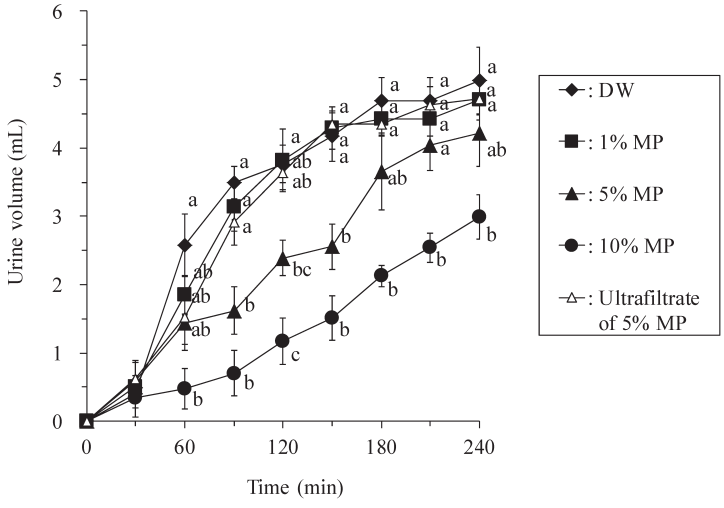

Fig. 1. Changes of Cumulative Urine Excretion after the Administration of Milk Protein Solutions and Its Ultrafiltered Solution

Rats were starved for 20 h and deprived of water for 4 h, followed by an oral administration of 6 mL of one of the test solutions. Their urine was collected and the urine volume was measured for 4 h. DW, distilled water; MP, milk protein. Each value is expressed as mean±S.E.M. (n=8–9). Values without a common letter differ significantly ($p<0.05$).

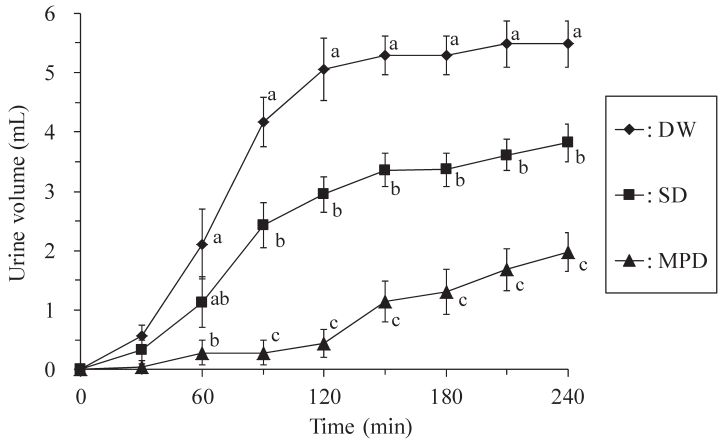

Fig. 2. Changes of Cumulative Urine Excretion after the Administration of Experimental Drinks

Urine volumes were measured as described in the Fig. 1 legend. DW, distilled water; SD, sports drink; MPD, milk protein-enriched drink. Each value is expressed as mean±S.E.M. (n=7). Values without a common letter differ significantly (p<0.05).

Table 2. Urinary Sodium Concentration and Excretion

	Unit	DW	SD	MPD
Urine volume	mL	5.48±0.39a	3.82±0.32b	1.98±0.33c
Urine sodium concentration	mg/mL	0.26±0.01a	0.47±0.06b	1.13±0.16c
Urine sodium excretion	mg	1.56±0.18	1.93±0.36	2.08±0.27

DW, distilled water; SD, sports drink; MPD, milk protein-enriched drink. Sodium concentration and excretion of urine accumulated for 4 h were measured. Each value is the mean±S.E.M. (n=7). Values without a common letter differ significantly (p<0.05).

Rate of water reabsorption (%)
= (1 − urine production rate (mL/min)/ glomerular filtration rate (mL/min)) × 100
≈ (1 − urine production rate (mL/min)/ creatinine clearance (mL/min)) × 100
= (1 − plasma creatinine (mg/dL)/ urine creatinine (mg/dL)) × 100

In this expression, creatinine clearance was used as an indicator of the glomerular filtration rate (GFR). GFRs were calculated with creatinine values in plasma and urine at 2 h after the test drink administration, when the volumes of urine excretion increased approximately linearly with time.

Experiment 3: Changes in Insulin after the Administration of the MPD The rats were starved for 20 h and were deprived of water for 4 h, followed by an oral administration of 6 mL of DW, SD, or MPD *via* a stomach sonde. Blood samples were collected from the saphenous vein at 0, 30, 60, 90, 120, 180, and 240 min after the administration and heparinized. Plasma was separated by centrifugation. The plasma insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Statistics All values are expressed as means with their standard errors. The homogeneity of variance was examined using Bartlett's test. When the variances were suspected to be equal among the experimental groups, the data were analyzed by the Tukey–Kramer test. If the variances were not expected to be equal, the data were analyzed by the Steel–Dwass test. Significance was defined as a p-value less than 0.05. These tests were performed using StatLight software (Yukms, Kawasaki, Japan).

RESULTS

Experiment 1: Relationship between the Milk Protein Concentration and the Fluid-Retention Effect The cumulative urinary volumes excreted up to each time point are shown in Fig. 1. The urinary volumes at 90 and 150 min in the 5% MP group were significantly lower than the corresponding values of the DW group. In the 10% MP group, the urine volumes at all time points from 60 to 240 min were significantly lower than the corresponding values of the DW group. No significant differences were observed between the 1% MP group and the DW group at any time points.

In addition, the urine volumes in the 5% MP group at 90 and 150 min were significantly lower than those of the group administered the ultrafiltrate of the 5% MP. No significant differences were observed between the ultrafiltrate of 5% MP group and the DW group at any time points.

Experiment 2: The Fluid-Retention Effect of the Milk Protein-Enriched Drink The cumulative urinary volumes excreted up to each time point are shown in Fig. 2. The urinary volumes in the MPD group at all time points from 90 to 240 min were significantly lower than the corresponding values of all of the other groups. The SD group's urinary volumes at all time points from 90 to 240 min were significantly lower than the corresponding values of the DW group.

The urinary sodium concentration and excretion values are shown in Table 2. The MPD group showed the highest concentration, followed by the SD group and the DW group in that order, with significant differences between all groups. The sodium excretion of the MPD group was at the same

Table 3. Water Weights in the Lumens of Stomach and Small Intestine 4 h after the Administration of the Test Drinks

	DW	SD	MPD
Stomach (g)	0.078±0.020a	0.236±0.032b	0.222±0.051b
Small intestine (g)	0.502±0.043	0.637±0.062	0.478±0.048

DW, distilled water; SD, sports drink; MPD, milk protein-enriched drink. Each value is the mean±S.E.M. (n=7). Values without a common letter differ significantly (p<0.05).

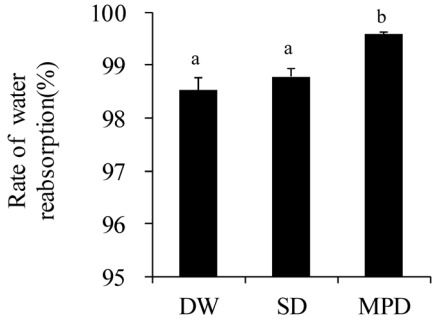

Fig. 3. Rate of Water Reabsorption after the Administration of Experimental Drinks

Water reabsorption rates in the renal tubules were calculated along with creatinine clearance and the urine production rate. Each value is expressed as mean±S.E.M. (n=7). Values without a common letter differ significantly (p<0.05).

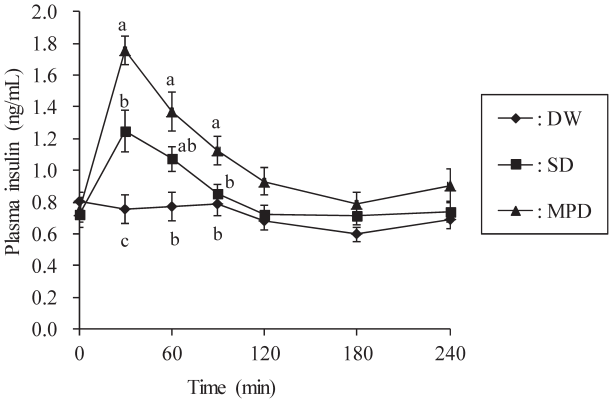

Fig. 4. Changes of Plasma Insulin after the Administration of the Three Experimental Drinks

Rats were starved for 20 h and deprived of water for 4 h, followed by an oral administration of 6 mL of one of the experimental drinks. Blood samples were taken sequentially and the plasma insulin concentration was measured. Each value is expressed as mean±S.E.M. (n=7). Values without a common letter differ significantly (p<0.05).

level as that of the SD group. These groups' sodium excretion values tended to be higher than those of the DW group, but a significant difference was not detected.

Table 3 provides the water weights in the lumens of stomach and small intestine. The water weights in the lumens of the stomach in the SD and MPD groups were significantly higher compared to that of the DW group (p<0.05), but these weights were lower compared to the volume of administration. In the small intestine, no significant differences were detected between any groups.

Figure 3 demonstrates the rates of water reabsorption in the renal tubule. The rate in the MPD group was significantly higher than those of the other groups (p<0.05).

Experiment 3: Insulin Change after the Administration of the Milk Protein-Enriched Drink We measured the plasma insulin after the administration of three test drinks (DW, SD, MPD) used in Experiment 2, and we found that the plasma insulin concentrations of the SD group and the MPD group increased after the administration and showed a peak at 30 min (Fig. 4). That of the MPD group was significantly higher than those of the other groups at 30 and 90 min (p<0.05).

DISCUSSION

In this study, we focused on the effect of milk protein on fluid retention, and based on our findings we propose an effective new oral rehydration method. We found that the addition of milk protein to a solution enhanced the fluid retention dose-dependently and that the rehydration drink containing approx. 5% milk protein was more effective for rehydration than a conventional protein-free sports drink.

In Experiment 1, we investigated the relationship between the concentration of the milk protein and the fluid-retention effect, with the goal of developing a new rehydration drink. The addition of milk protein at the concentrations of 5 and 10% reduced the rats' urinary volumes in a dose-dependent manner (Fig. 1). This result indicates that milk protein has the dose-dependent effect of fluid retention and that the minimum effective concentration is approx. 5%. The milk protein concentrate used in this study contains small amounts of sodium and carbohydrates which have the effect of fluid retention[4,5]; however, the results obtained with the ultrafiltrate of 5% MP (Fig. 1) demonstrate that the sodium and carbohydrates contained in the milk protein solution hardly contribute to the fluid retention.

Based on these data from Experiment 1, we prepared a milk protein-enriched drink (MPD) that contained approx. 5% milk protein, and we assessed the fluid-retention effect of the MPD in Experiment 2. The results (Fig. 2) indicate that the fluid-retention effect of the MPD was clearly higher than that of the conventional sports drink tested.

The water content of each test drink differed slightly. Those of the SD and MPD were 96% and 92% (v/v), respectively. In contrast, the urine excretion values differed largely among the test drinks, and we infer that the impact of the difference in the water content among the test drinks is quite small. We performed an additional experiment measuring the impact of the difference in water content: we compared the urine volume of rats administered 6 mL DW with that of rats administered 5.5 mL DW (5.5 mL DW corresponds to the water content in the MPD group). The urine volumes after 240 min in the 6 mL DW group and 5.5 mL DW group were 5.1 mL and 4.8 mL, respectively, with no significant difference (data not shown).

If the water ingested by the rats stayed in their gastrointestinal tracts, the water could not be used immediately for controlling body temperature by the promotion of sweating or skin blood flow. In Experiment 2, the weights of the water

that remained in the lumens of the stomach and the small intestine were notably lower than the volume of administration in all groups (Table 3). Therefore, it was shown that most of the water was absorbed at the latest by 4 h after the test-drink administration in the MPD group as well as in the DW and SD groups.

We also measured the changes in the plasma volume to confirm whether the water in each test drink was absorbed from the intestine and stayed in the blood after administration. The plasma volumes increased 4 h after the oral administration in all groups, but no significant difference was detected between any groups (data not shown). One of the reasons for this result may be that the absorbed water contributes to the increase not only of the plasma volume but also of the interstitial fluid volume. Incidentally, it was suggested that a moderate increase in the interstitial fluid volume can prevent dehydration because the interstitial fluid can act as a reservoir for plasma water. That is, water can move from the interstitial fluid to the plasma when the plasma volume is decreased by dehydration.[14,15] An excess of interstitial fluid causes edema. We speculate that the new MPD described herein can be ingested safely because no abnormalities such as edema were observed in any of the rats' organs at autopsy. However, further studies are needed to precisely determine how the volumes of body fluids such as plasma, interstitial fluid and intracellular fluid change after ingestion of the MPD.

It is thought that the regulation of the reabsorption of water and sodium in the kidneys strongly relates to the mechanism of fluid retention. We thus estimated the rate of water reabsorption in the kidneys after the administration of the test drinks, and the results showed that the water reabsorption rate after the ingestion of the MPD was higher than that of the DW group (Fig. 3). In contrast, it seems unlikely that sodium reabsorption was promoted after ingestion of the MPD because the excretion and the ingestion of sodium in the MPD group were at the same level as those of the SD group (Tables 1, 2). We therefore suggest that the contribution of water reabsorption to the MPD's fluid-retention effect is larger than that of sodium reabsorption.

The difference in the water reabsorption rates between the DW and MPD was approx. 1%. This difference could greatly affect the urine volume. For example, in a previous study of dogs administered diuretics, the change in the water reabsorption rate was 0.57% when the urine volume increased more than twofold.[16]

Our findings confirmed that the concentration of plasma insulin in the MPD group was higher than those in the other groups (Fig. 4). The MPD's strong effect on the secretion of insulin may be explained by the report that the ingestion of milk protein promotes the secretion of insulin.[17] According to previous *in vitro* research, insulin is thought to have a role in the regulation of water reabsorption in the kidneys in addition to its role in glucose metabolism. The results of studies using rabbit proximal convoluted tubule[18] and rat inner medullary collecting duct[19] suggest that insulin can promote water reabsorption in the kidneys. Additionally, insulin is reported to promote the expression of the protein aquaporin 2, on which water absorption in the kidneys depends.[20] It is thus likely that in the present MPD group the insulin secretion after the ingestion of milk protein contributed to the high rate of water reabsorption.

Future research must be conducted regarding the mechanisms underlying the fluid-retention effect of milk protein-enriched drinks, and a human study is also needed to examine these drinks' effectiveness for preventing dehydration.

In conclusion, we found that increasing the milk protein concentration in a drink enhanced fluid retention, a result which may make it possible to develop more effective rehydration drinks than the conventional protein-free sports drinks. In addition, insulin-modifying renal water reabsorption may contribute to the fluid-retention effect of the MPD tested here.

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Conflict of Interest Kentaro Ito, Yuri Saito, Kinya Ashida, Taketo Yamaji, and Hiroyuki Itoh are employees of Meiji Co., Ltd. Munehiro Oda has no conflict of interest.

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